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Blastula-Stage Stem Cells Can Differentiate into Dopaminergic and Serotonergic Neurons after Transplantation

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In order to assess the potential of embryonic stem cells to undergo neuronal differentiation *in vivo*, totipotent stem cells from mouse blastocysts (D3 and E14TG2a; previously expanded in the presence of leukemia inhibitory factor) were transplanted, with or without retinoic acid pretreatment, into adult mouse brain, adult lesioned rat brain, and into the mouse kidney capsule. Intracerebral grafts survived in 61% of cyclosporine immunosuppressed rats and 100% of mouse hosts, exhibited variable size and morphology, and both intracerebral and kidney capsule grafts developed large numbers of cells exhibiting neuronal morphology and immunoreactivity for neurofilament, neuron-specific enolase, tyrosine hydroxylase (TH), 5-hydroxytryptamine (5-HT), and cells immunoreactive for glial fibrillary acidic protein. Though graft size and histology were variable, typical grafts of 5–10 mm³ contained 10–20,000 TH⁺ neurons, whereas dopamine-β-hydroxylase⁺ cells were rare. Most grafts also included nonneuronal regions. In intracerebral grafts, large numbers of astrocytes immunoreactive for glial fibrillary acidic protein were present. Both TH⁺ and 5-HT⁺ axons from intracerebral grafts grew into regions of the dopamine-lesioned host striatum. TH⁺ axons grew preferentially into striatal gray matter, while 5-HT⁺ axons showed no white/gray matter preference. These findings demonstrate that transplantation to the brain or kidney capsule can induce a significant fraction of totipotent embryonic stem cells to become putative dopaminergic or serotonergic neurons and that when transplanted to the brain these neurons are capable of innervating the adult host striatum. © 1998 Academic Press

Key Words: embryonic stem cells; brain tissue transplantation; cell development; dopamine; serotonin; Parkinson's disease; Lesch-Nyhan disease.

INTRODUCTION

Experimental model systems involving pleuripotent progenitor cells provide data for analysis of factors that influence and control development to a neurally restricted cell fate (32, 51, 53, 54). Pleuripotent cell lines used in studies of neural differentiation can be roughly divided into four general categories. A subset of neural progenitors can be growth factor-expanded (GFE) *in vitro* in the presence of basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF). GFE cell lines can be expanded in culture without the use of genetic manipulation and have been derived from embryonic (6, 26, 28, 35, 40, 42) and adult (15, 43, 44) rodent brain regions, as well as from human embryonic brain (52). Immortalized primary neural (IM) cell lines, produced from cells in the developing CNS immortalized with an oncogene, such as v-myc or the temperature-sensitive mutant of SV40 large T antigen, also provide an expandable multipotent cell source (12, 46). The brainstem-derived IM cell line RN33B demonstrates the capacity for IM cells to differentiate into neurons in a site-specific manner when grafted into adult brain (29, 37, 49). Embryonal carcinoma (EC) cells derived from germ cell- or teratocarcinoma tumors can also be induced to differentiate into multiple CNS cell types (30, 31, 39).

Embryonic stem (ES) cell lines derived from the inner cell mass of preimplantation mouse embryos are potentially capable of reconstituting all cell types of the body (3, 9, 36), thereby allowing the study of all steps involved in development. Totipotent ES cells can be maintained in an undifferentiated, proliferative state in the presence of leukemia inhibitory factor (LIF). When LIF is removed, these ES cells differentiate into a variety of cell types *in vitro*, both spontaneously and via inductive agents. Recently, treatment of ES cells *in vitro* with retinoic acid (RA) has shown that a high proportion can be induced to differentiate into neurons (2, 7, 11). In subsequent experiments investigating intracerebral grafts of ES cells, we found that a large number of catecholaminergic cells were produced even without pretreatment with RA. To investigate factors contributing to the *in vivo* development of totipotent ES cells into these neuronal phenotypes, we compared four

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variables: ES cell genotype (wild-type versus hypoxanthine-guanine phosphoribosyltransferase deficient; HPRT-deficient), pretreatment of ES cells with or without RA, differences in transplantation site (brain and kidney capsule), and differences in host species (adult mouse versus rat). To specifically assess the capacity of ES cells to differentiate into monoaminergic neurons, we analyzed the transplanted tissue for presence of serotonergic (5-HT$^+$) and dopaminergic (TH$^+$ and DβH$^-$) cells and for axonal outgrowth into rat striatum previously denervated of DA fibers.

METHODS

ES cell preparation. The mouse blastocyst-derived ES cell lines D3 and E14TG2a (A.T.C.C.; Rockland, MD) were used for all studies (8, 10); the E14TG2a line was HPRT-deficient. Undifferentiated ES cells were maintained on gelatin-coated dishes in Dulbecco's modified Minimal Essential Medium (DMEM, Gibco/BRL, Grand Island, NY) supplemented with 2 m*M* glutamine (100× stock from Gibco/BRL), 0.001% β-mercaptoethanol, 1× nonessential amino acids (100× stock from Gibco/BRL), 10% donor horse serum (HyClone, Logan, UT), and human recombinant leukemia inhibitory factor (LIF; R & D Systems, Minneapolis, MN) (7). Early passage cultures were frozen (90% horse serum/10% DMSO), thawed for use, and cultured for 2 weeks in the presence of LIF. Cells were trypsinized (0.05% trypsin-EGTA; GIBCO), resuspended, then seeded at 1.5×10^6 cells in 5 ml of DMEM + 0.5 m*M* retinoic acid (RA$^+$) (Sigma Chemical Co., St. Louis, MO) or in the same media without RA (RA$^-$) in a 60-mm Fisher brand bacteriological grade petri dish, in the absence of LIF. Horse serum was replaced by 10% fetal calf serum (FCS; Hyclone) during this treatment. ES cells did not adhere to the dish but formed small aggregates. After 2 days of incubation at 37°C, the cells were transferred to a 15-ml sterile culture tube and allowed to settle, and the media was replaced with an equal volume of fresh RA$^+$ or RA$^-$ media. The cells were then replated and incubated for an additional 2 days. After 4 days, cells were collected and rinsed once in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (D-PBSa, Gibco/BRL). D-PBSa was removed, 0.5 ml of trypsin solution was added, and the cells were incubated for 5 min at 37°C, then triturated with a pasteur pipette to dissociate the cells. The trypsin solution was replaced with 0.1 *M* phosphate-buffered saline, pH 7.4 (PBS), and viability was determined by the acridine orange-ethidium bromide method (4): viability of cells after removal from the culture dish was greater than 95% in all cases. ES cells derived directly from monolayers after LIF removal were also implanted in some cases, following the above procedures minus the incubation steps. No systematic difference due to incubation time was observed in the resulting grafts and so RA$^-$ cases are pooled in this report.

ES cell transplantation. Sprague–Dawley rats (300–350 g) and C57/B15 mice (14–17 g) (Charles River Labs, MA) were used as intracerebral-transplant recipients. Cell concentrations and dosages varied in different experiments: rat hosts received from 100,000 to 300,000 viable ES cells per right striatum (60,000–100,000 viable cells/μl), and mice received 60,000 ES cells per right striatum (60,000 viable cells/μl). For all neural surgical procedures, animals were anesthetized with pentobarbital (65 mg/kg, i.p.), and placed in a Kopf stereotaxic frame (with Kopf mouse adapter for mice). Mice ($n = 7$) used as intracerebral transplant hosts were normal adult females, and rats ($n = 31$) used as transplant hosts were adult females that had received prior unilateral nigrostriatal 6-hydroxydopamine (6-OHDA) lesion removing at least 97% of DA innervation, as previously described (14). ES cells were implanted stereotaxically (from Bregma: A+ 1.0 mm, L −2.5 mm, V −4.5 mm, IB −2.5 mm). A 10-μl Hamilton syringe attached to a 22S-gauge needle (ID/OD 0.41 mm/0.71 mm) was used to deliver 1 μl (mouse) or 3–5 μl (rat) of ES cell suspension (rate: 1 μl/min, allowing an additional 2 min for the final injection pressure to equilibrate before slowly withdrawing the injection needle). Starting on the day prior to transplantation, rats were immunosuppressed with Cyclosporine-A (10–15 mg/kg, s.c. daily) for the duration of the experiment to prevent graft rejection. Mice were not immunosuppressed. Nude mice (Charles River) were used as kidney-capsule transplant recipients. Mice were anesthetized (as above), and 50,000 ES cells (in 1 μl), not pretreated with RA, were injected into a blood clot derived from host blood; this clot was then implanted unilaterally into one kidney capsule ($n = 3$ with E14TG2a line and $n = 3$ with D3 line).

Histological procedures. Two or four weeks after transplantation, animals were terminally anesthetized (pentobarbital; 100 mg/kg), then perfused intracardially with 100 ml heparin saline (0.1% heparin in 0.9% saline), followed by 400 ml of paraformaldehyde (4% in PBS). The brains or kidney capsules were removed and postfixed for 8 h in the same 4% paraformaldehyde solution. Following postfixation, the brains and kidney capsules were equilibrated in sucrose (30% in PBS), sectioned (40 mm) on a freezing microtome, and collected in PBS. Sections were divided into 6–8 series and stored in PBS at 4°C. Separate series were processed for either Nissl staining (cresyl violet acetate), or acetylcholinesterase (AChE) histochemistry (as described in (38). Immunohistochemical markers used for tissue processing included antibodies directed against neuron-specific enolase (NSE, Dako, Carpenteria, CA), mouse-specific Thy 1.1 (Clone TN-26, Sigma), tyrosine hydroxylase (TH; PelFreez, Rogers, AK), 5-hydroxytryp-

tamine (5-HT, Arnel Products, New York, NY), 200- + 68-kDa neurofilament (NF, Biodesign, Kennebunkport, ME), dopamine-β-hydroxylase (DβH; Chemicon, Temecula, CA), proliferating cell nuclear antigen (PCNA; Chemicon), and glial fibrillary acidic protein (GFAP; Boehringer-Mannheim).

Free floating tissue sections were pretreated with 50% methanol and 3% hydrogen peroxide in PBS for 20 min, washed three times in PBS, and incubated in 10% normal goat serum (NGS) in PBS for 60 minutes prior to overnight incubation on a shaking platform with the primary antibody. After a 10-min rinse in PBS and two 10-min washes in 5% NGS, sections were incubated in biotinylated secondary antibody (goat-anti-rabbit or goat-anti-mouse, depending on primary species) at a dilution of 1:200 in 2% NGS in PBS at room temperature for 60–90 min. The sections were then rinsed three times in PBS and incubated in avidin–biotin complex (Vectastain ABC kit ELITE; Vector Labs) in PBS for 60–90 min at room temperature. Following thorough rinsing with PBS and Tris-buffered saline, sections were developed for 5–30 min in 0.04% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine (Sigma) in Tris-buffered saline. Controls with omission of the primary antibody were performed on selected sections to verify the specificity of staining. After immunostaining, floating tissue sections were mounted on glass slides, coverslipped, and analyzed with bright and darkfield light microscopy using a Zeiss Axioplan microscope. Quantitative analyses were performed with the aid of NIH Image software (Ray Rasband, NIH, Bethesda, MD) and cell counts from serial sections were corrected and extrapolated for whole graft volumes using the Abercrombie method (1). Selected images were digitized using a Leaf Lumina video scanning camera (Leaf Systems, Newton, MA) into Adobe Photoshop which was used to prepare and print final figures.

RESULTS

Neuronal Differentiation within Intracerebral ES Cell Grafts

ES cells with (RA^+) or without (RA^-) retinoic acid pretreatment were implanted into rodent striatum. In animals receiving RA^+ ES cells grafts, surviving transplants were found in 2/5 immunosuppressed rat hosts and 4/4 mouse hosts. In animals receiving RA^- ES cell grafts, surviving transplants were found in 17/26 immunosuppressed rats and 3/3 mice. Graft sizes and overall histological appearance varied greatly between animals: mean graft volumes were in the order of 5–10 mm^3, the smallest grafts were in the order of 2–3 mm^3, while grafts in some rat hosts were very large (in the order of 30 mm^3) and filled or expanded beyond the striatal boundary (1 of the 2 RA^+ ES grafts and 12 of the 17 RA^- ES grafts). The largest grafts exhibited complex, heterogeneous tissue types with significant fractions of graft volume that were negative for neuronal markers and also contained regions which stained positive for proliferating cell nuclear antigen (PCNA; data not shown). All 7 of the mouse hosts had smaller grafts, none of which exhibited complex, heterogeneous tissue types.

No consistent correlations were observed in intracerebral graft morphology and graft cell types with respect to presence or absence of lesion or host species. Mouse cell-specific staining with Thy 1.1 in rat hosts (Fig. 1A) indicated that donor cells did not migrate away from the site of implantation, but formed a localized cell-dense graft. Even without RA pretreatment, ES cell grafts contained large areas immunoreactive for NF (Fig. 1B) and NSE (Fig. 1C). All ES cell grafts exhibited a slightly higher cell density than the surrounding host striatum, as indicated by Nissl staining (Fig. 2A). RA^+ or RA^- ES cell grafts had similar overall appearance, but RA^+ grafts appeared to have more AChE staining (Fig. 2B) than RA^- grafts (Fig. 3A).

Presence of Monoaminergic Neurons and Glial Cells within Intracerebral ES Cell Grafts

In 22 of the 26 surviving intracerebral grafts, regardless of RA pretreatment, host species, or graft size, a number of TH^+ cells with neuronal morphologies and neural processes were observed (Figs. 2C and 3B). Sections from 16 animals containing grafts were immunoreacted for 5-HT, and all contained $5\text{-}HT^+$ cells with neuronal morphology (Fig. 3C) including extensive neural processes. Grafts contained numerous dense monoaminergic cell clusters interspersed with regions that did not stain for these markers (Figs. 2C, 3B, 3C, 5A, and 5B). Most grafts exhibited very large numbers of TH^+ (Fig. 5A) and $5\text{-}HT^+$ (Fig. 5B) neurons. For example, one typical graft of intermediate size (S9-N1; 6.02 mm^3) contained approximately 14,450 TH^+ neurons (using Abercrombie correction) (1). In order to determine whether the TH^+ cells were noradrenergic/adrenergic or dopaminergic, additional sections were immunoreacted for DβH; 14 of 15 ES cell grafts were negative for DβH, thus the majority of TH^+ cells were presumed to be of the DA neurotransmitter phenotype (Fig. 3D). In only one graft, there were a few lightly stained DβH cells. The presence of $D\beta H^+$ host noradrenergic axons visible in the cerebral cortex of the same sections served as positive controls. In comparing the two ES cell lines, D3 and E14TG2a (HPRT-deficient), similar development of TH^+ neuronal cells was seen (Figs. 4A and 4B).

Sections containing grafts were also immunoreacted for the presence of glial fibrillary acidic protein (GFAP). ES cell grafts were densely filled with GFAP-positive cells and fibers, and GFAP+ cell-dense regions corresponded to neuron-rich regions of the grafts in adjacent

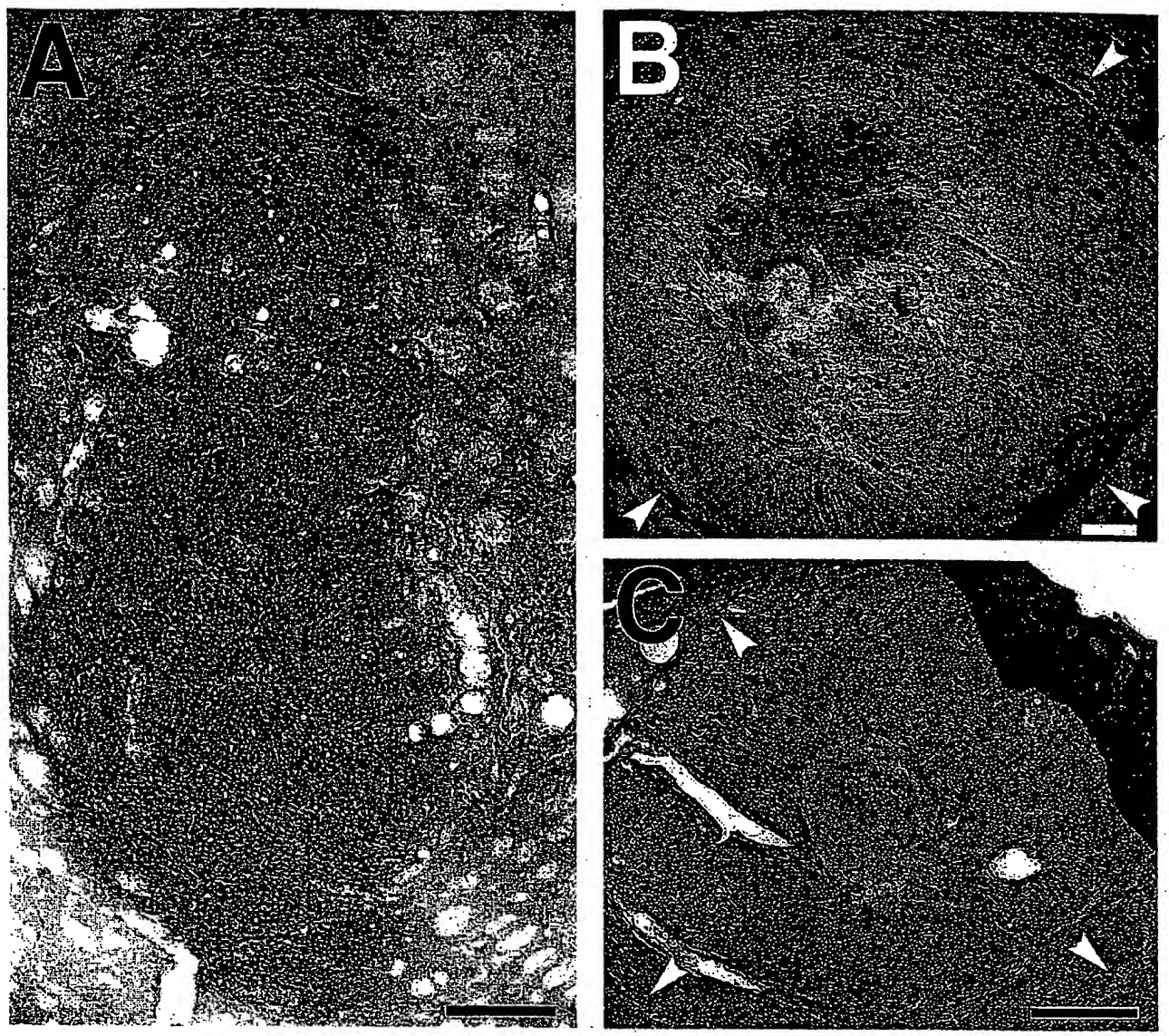

FIG. 1. Three intracerebral mouse ES cell grafts in the rat striatum showing immunoreactivity for markers of mouse cells (using an antibody to Thy 1.1; shown in A), neurofilaments (using an antibody to 65- and 200-kDa neurofilament; shown in B), and neurons (using an antibody to neuron-specific enolase; shown in C). Thy1.1 immunoreactivity (dark stain in A) demonstrates that the vast majority of grafted ES cells remain within the region of the implantation site. Neurofilament immunoreactivity (bright fibers in B) demonstrates that a large fraction of the graft volume is filled with neuronal processes, though there may be regions within these grafts that are neurofilament negative (dark region in the center of the graft). NSE immunoreactivity (dark staining in C) indicates that a large fraction of the graft volume contains neuronally differentiated cells, though regions that contain few such cells can also be identified (such as the light region in the center of the graft). The dark band in the upper right of this graft is an artifact of tissue folding along the ventricle. Graft boundaries are indicated by arrows in B and C. Scale bar in A, 200 μm; in B, 200 μm; in C, 200 μm.

sections (data not shown). The surrounding host brain also contained GFAP+ cells, but in lower density that diminished with distance from the graft.

Host Striatal Innervation by Monoaminergic Axons from ES Cell Grafts

Immunohistochemistry for TH and 5-HT in rat hosts that were unilaterally lesioned with 6-OHDA enabled assessment of axonal outgrowth into surrounding host tissues. TH^+ axons from both RA^+ and RA^- ES cell grafts extended into the DA-denervated host striatum (Fig. 6A). These axons were of significantly larger caliber and lower density than host DA axons in the unlesioned contralateral host striatum (Figs. 6C and 6D). In two graft cases, donor axons were observed extending as far as the striatal boundary with the

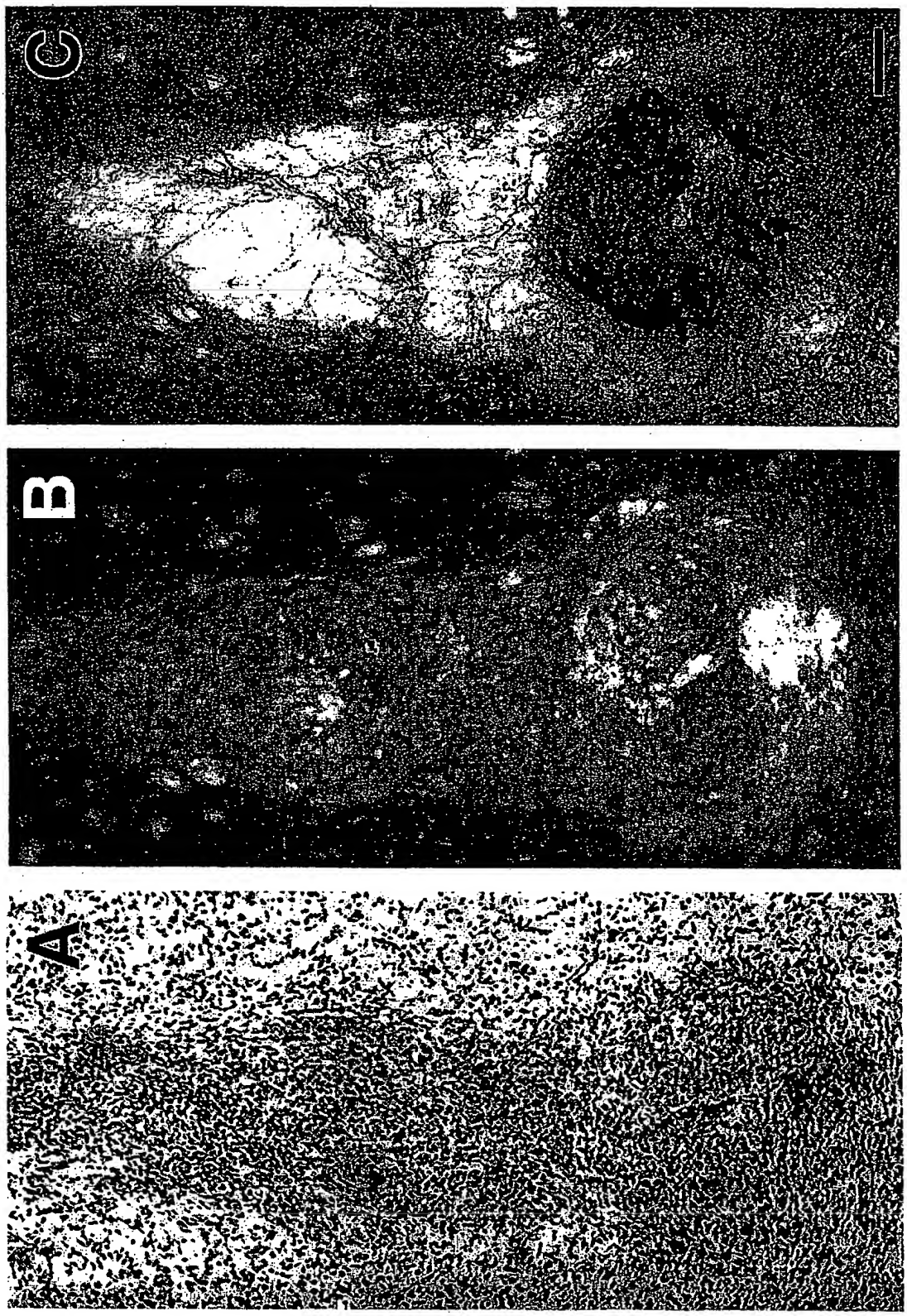

FIG. 2. An RA$^+$ ES cell graft 1 month after implantation into the mature mouse striatum. (A) Nissl stain shows ES cells aggregated in the grafted region. (B) AChE histochemistry is positive but weaker than seen in adjacent host striatum. (C) TH immunoreactivity identifies a region of the graft that is filled with TH$^+$ cells and processes. Scale bar, 100 μm.

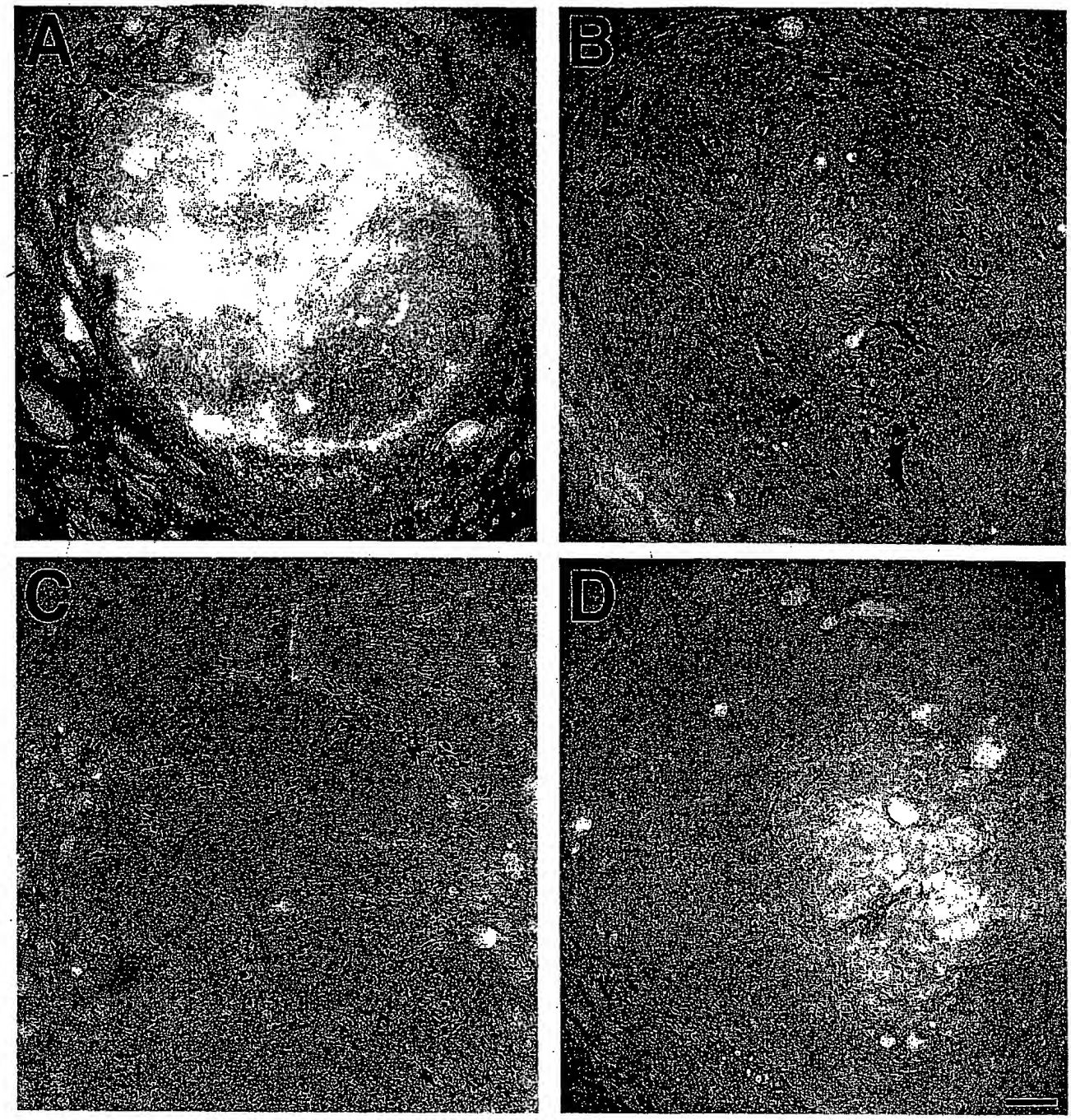

FIG. 3. An RA⁻ ES cell graft in the striatum of a rat host 1 month after implantation. (A) Cholinesterase histochemistry of this graft demonstrates weak activity. (B) TH immunoreactivity shows numerous TH⁺ cells irregularly distributed. (C) 5-HT (serotonin) immunoreactivity also labels cells distributed throughout the graft. (D) DβH immunoreactivity (a marker for noradrenergic and adrenergic cells) did not label grafted neurons, while host noradrenergic axons were identified in the opposite cortex in the same section (data not shown). Scale bar, 200 μm.

corpus callosum (>500 μm). TH⁺ axons grew preferentially into host striatal gray matter, avoiding corticofugal white-matter tracts of the striatum (Figs. 6A and 6C). Individual TH⁺ cells within the grafts exhibited robust dendritic trees (Figs. 4A and 4B); varicosities were commonly observed on axons contained within the graft body (Fig. 6B), but were absent or considerably reduced in size on graft axons that extended into the host striatum.

Axons from 5-HT⁺ graft cells were abundant in ES

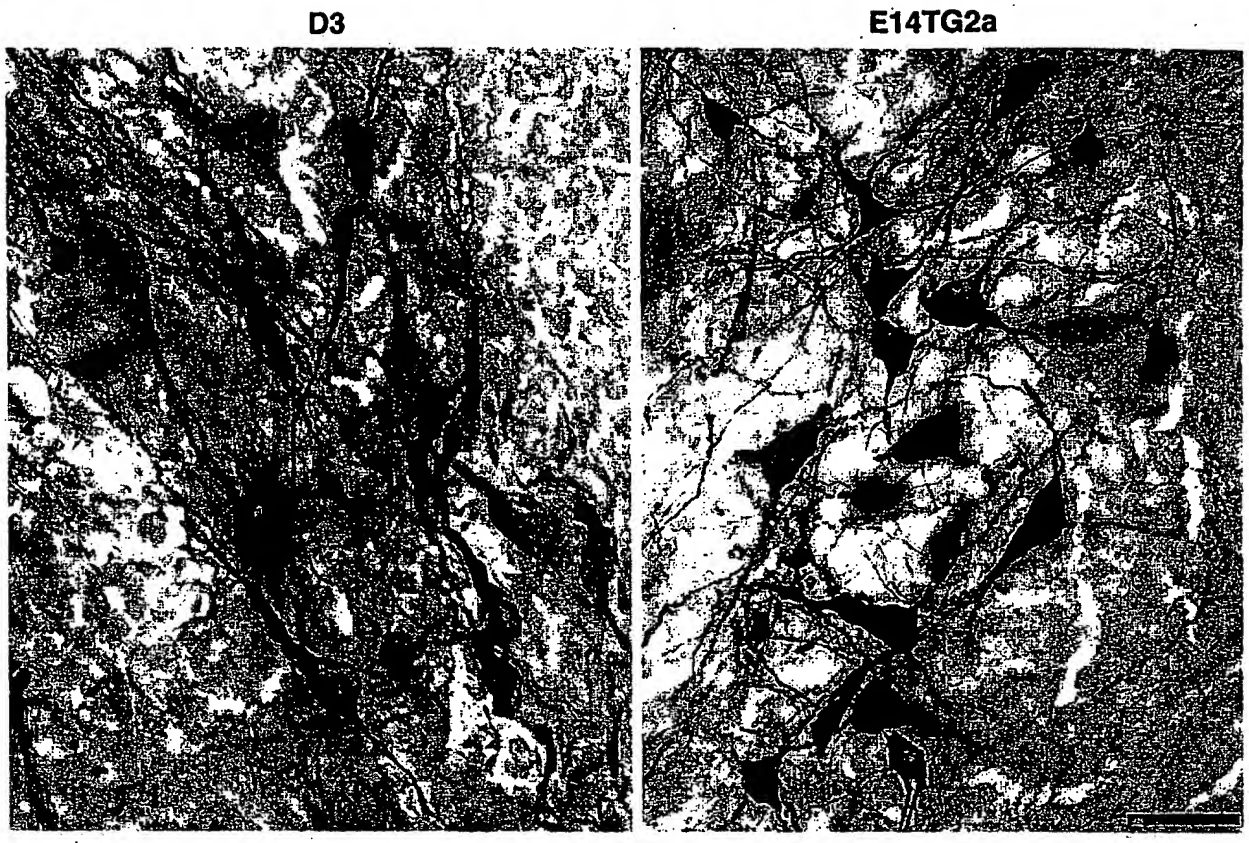

FIG. 4. TH+ cells within intracerebral grafts derived from either the D3 cell line (left panel) or the E14TG2a cell line (right panel). Scale bar, 20 μm.

cell grafts and extended into the host striatum (Figs. 7A and 7B). In the DA-denervated host striatum, 5-HT+ axons filled the graft and were primarily found a short distance (<500 μm) from the graft. The density of these axons was considerably higher than the host 5-HT innervation in the contralateral striatum (data not shown). In contrast to TH+ graft axons, 5-HT+ axons were distributed equally in striatal gray and white matter (Figs. 7A and 7B). The morphology of 5-HT+ neurons within these grafts also differed from the TH+ cells and axons: 5-HT+ neurons had less robust dendritic trees than TH+ neurons and lacked axonal varicosities (Fig. 7C, and compare Figs. 4A and 4B).

Monoaminergic Neurons Are Also Produced in ES Cell Grafts Placed in the Kidney Capsule

In order to determine whether the induction of these neuronal phenotypes could occur outside the CNS, we transplanted ES cells (RA−) to the mouse kidney capsule. Interestingly, the overall graft appearance and cell types were similar to those observed in intracerebral ES cell grafts. ES cell grafts in the kidney capsule were large, complex, and heterogeneous, as previously described for intracerebral ES cell grafts (see above). NF+ regions were seen throughout the grafts, and large numbers of TH+ and 5-HT+ cells with well-differentiated neuronal morphologies were also observed. These cells extended neurites within the ES cell grafts (Figs. 5C and 5D).

DISCUSSION

When transplanted into either CNS or non-CNS sites, blastula-derived ES cells of two different genotypes, in the presence or absence of RA pretreatment, can develop into heterogeneous tissues with many cells expressing neuronal markers and morphologies of mature neurons. Large numbers of TH+ and 5-HT+ neurons were found in grafts placed either in the brain or the kidney capsule. Large numbers of GFAP+ cells were observed in intracranial ES cell grafts as well as in surrounding host striatum, but there were also graft regions that were not immunoreactive for any of the neural markers used in this study.

Neural Differentiation of Grafted ES Cells

Large numbers of TH+ and 5-HT+ neurons were produced in these ES cell grafts (a typical graft of 6

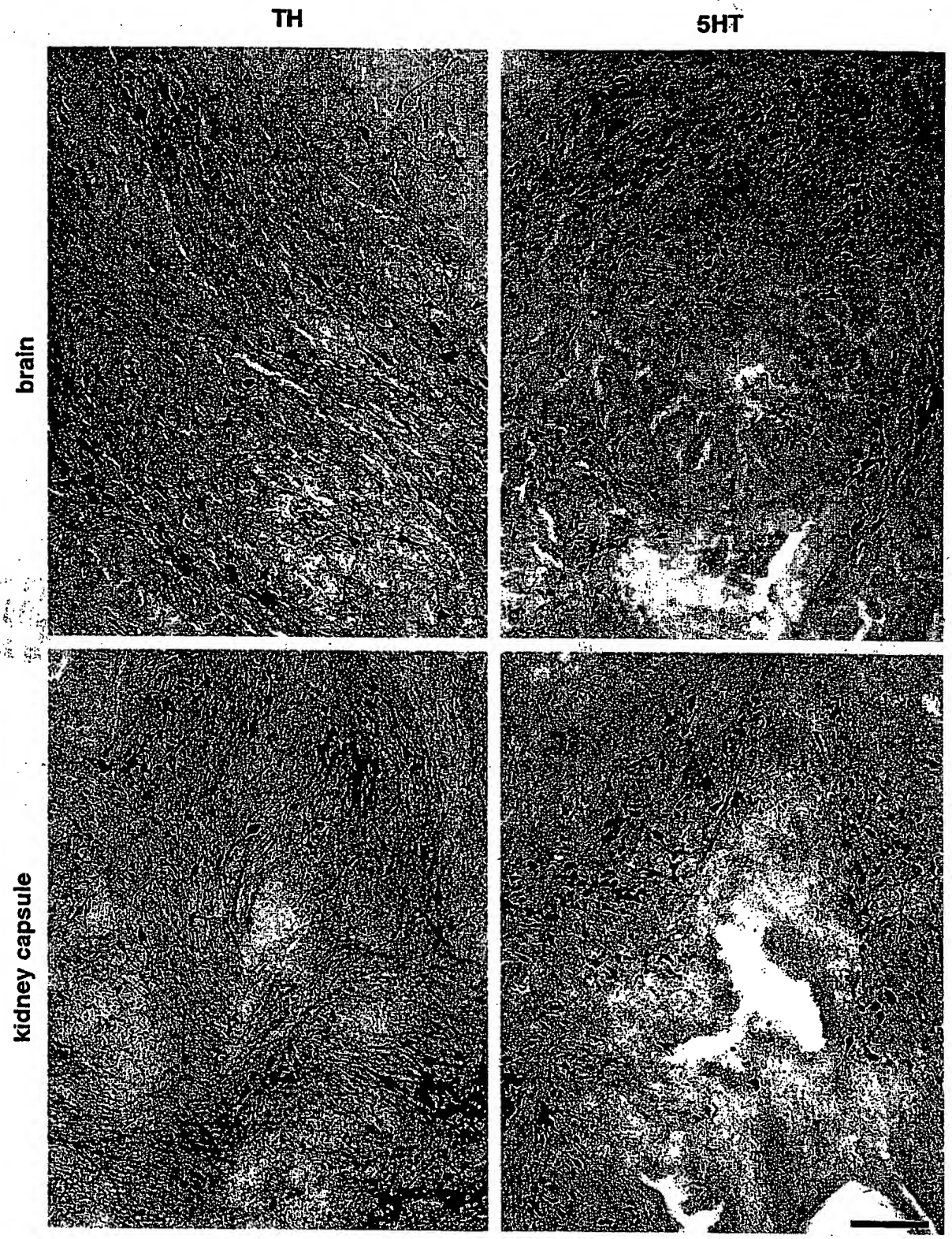

FIG. 5. TH$^+$ (left panels) and 5-HT$^+$ (right panels) cells from an uninduced ES cell graft placed in the rat striatum (upper panels) or in mouse kidney capsule (lower panels) 1 month after implantation. Both monoaminergic cell types are present in abundance in all grafts. These monoaminergic cells are distributed in dense patches and exhibit typical neuronal morphologies, including extensive neurite outgrowth. Scale bar, 100 μm.

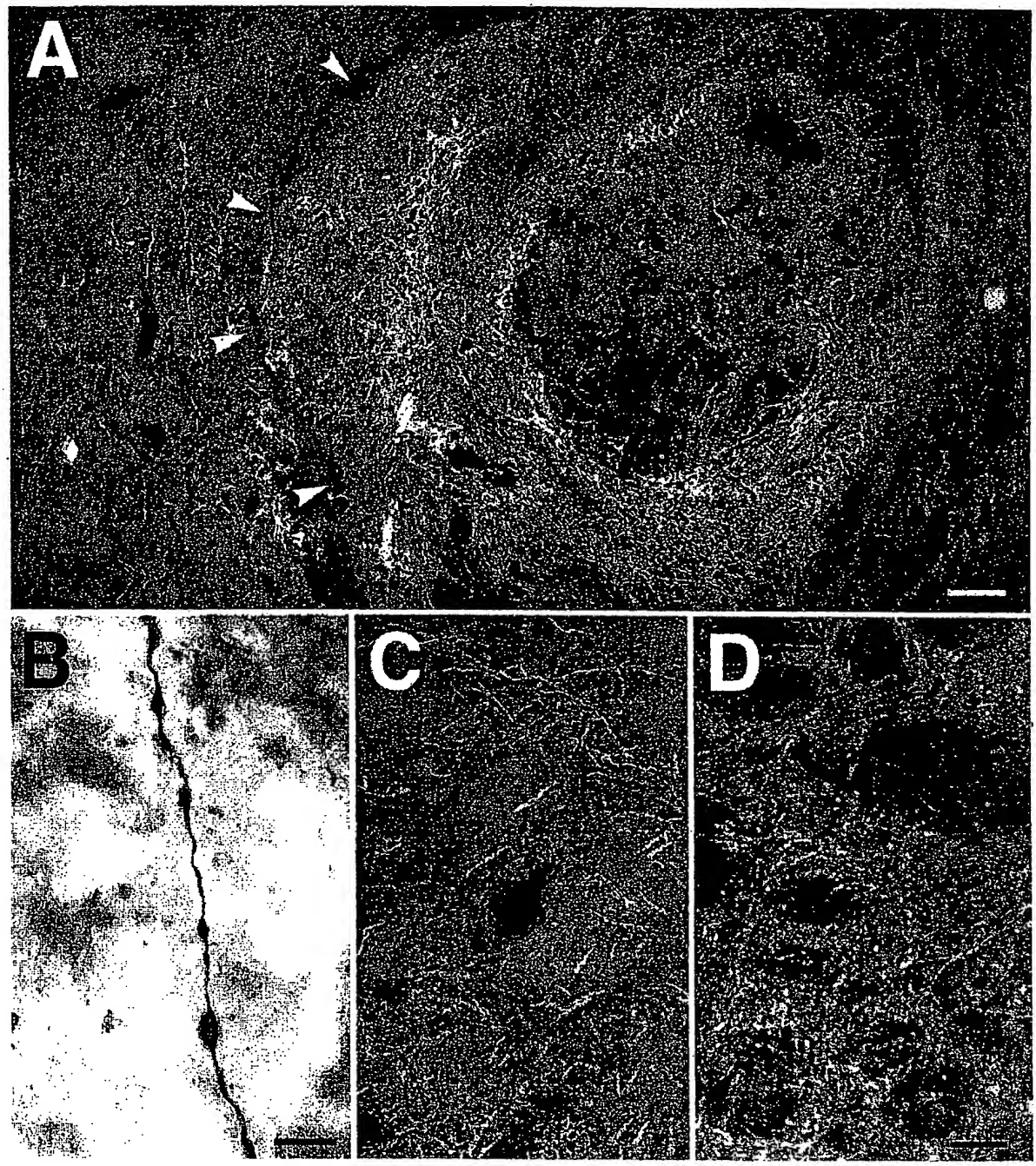

FIG. 6. Distribution of TH^+ neurons and axons from an intrastriatal RA^- ES cell graft placed in a DA-denervated rat striatum. (A) Darkfield image showing numerous TH^+ cells (brown) and axons (yellow) growing into the host striatum. The graft exhibits concentric rings of dense TH^+ cells and a central region that is relatively devoid of TH^+ somata. Arrows indicate graft–host boundary. (B) A high magnification brightfield image of a TH^+ axon *within* the graft exhibiting varicosities along its length. (C) Higher power darkfield image of TH^+ axons (bright fibers) growing within the gray matter of the host striatum. TH^+ axons are most dense in striatal gray matter and avoid myelinated fiber bundles, as is typical of the normal TH^+ fibers in the opposite unlesioned untransplanted hemisphere (shown in D). Scale bars: A, 250 μm; B, 10 μm; C and D, 50 μm.

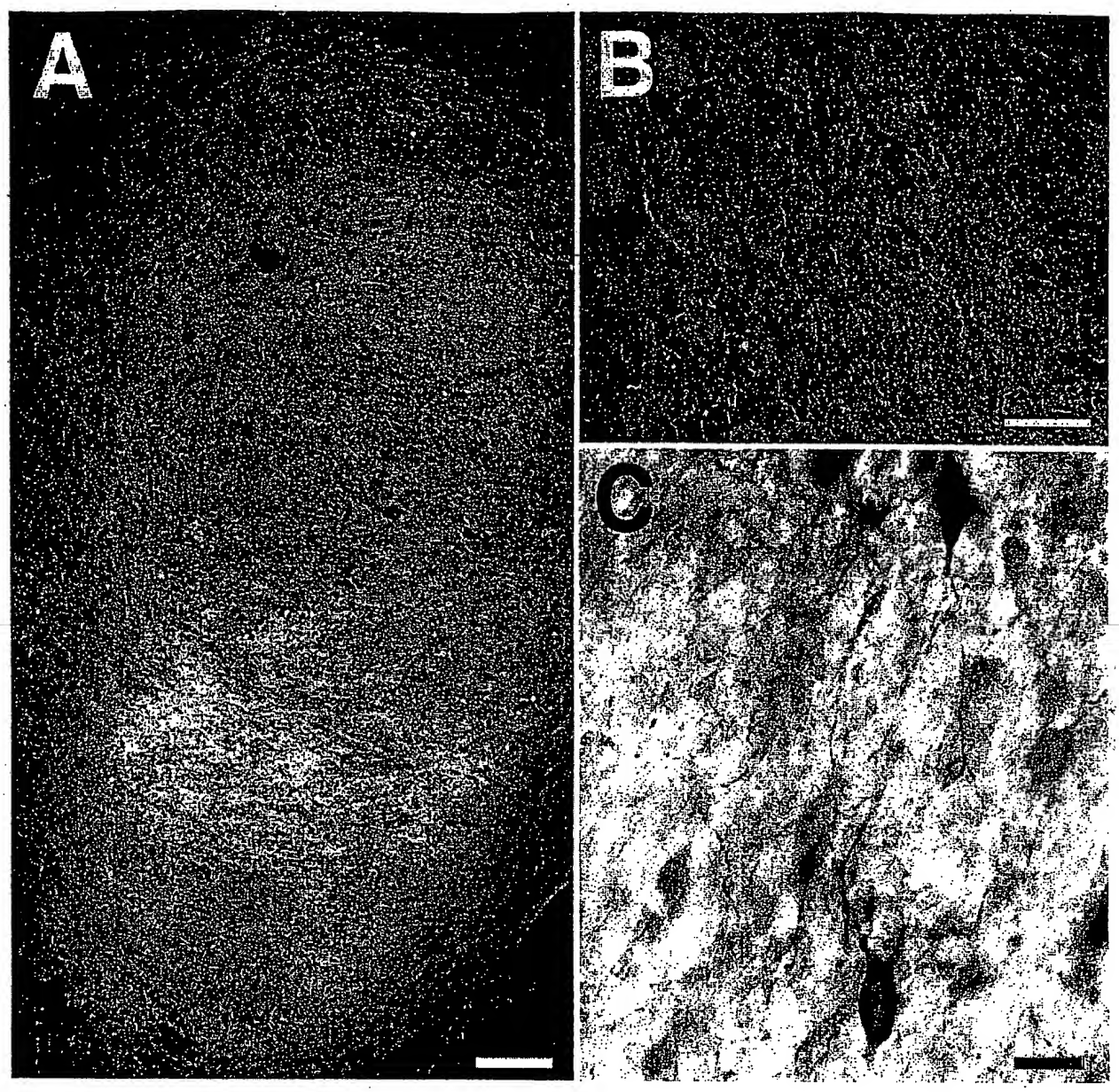

FIG. 7. (A) Darkfield image showing 5-HT$^+$ axons densely filling the graft region and extending into the host striatum. (B) Higher power image of the graft-host boundary on the left side, demonstrating fiber outgrowth. (C) Brightfield image of 5-HT$^+$ ES cell somata 1 month after implantation into the adult host striatum, demonstrating neuronal morphology. Scale bar in A, 200 μm, in B, 100 μm, and in C, 20 μm.

mm^3 contained on the order of 10–20,000 TH$^+$ and 5-HT$^+$ cells each). The rarity of DβH$^+$ cells with neuronal phenotypes further suggests that the TH$^+$ cells in these grafts are likely to be dopaminergic rather than adrenergic or noradrenergic. Moreover, the morphologies of these monoaminergic cells suggest that they may be homologues of CNS dopaminergic and serotonergic neurons. However, since double-labeling for TH and DβH was not performed, the presence of other catecholaminergic cell types cannot be ruled out. Verification of a dopaminergic phenotype awaits further assessment by histochemical, physiological, and behavioral assays. Both of these monoaminergic neuronal cell types extended axons into the graft and into the surrounding host brain. TH$^+$ graft axons grew preferentially into gray matter of the DA-denervated rat striatum, as is typical of normal striatal dopaminergic innervation. This specific innervation pattern has also

been observed for dopaminergic axons growing into the host striatum from fetal ventral mesencephalic grafts, but is not exhibited by nondopaminergic fibers from the same grafts (14, 22, 23), nor by axons from fetal cortical or striatal grafts to adult striatum (22). In contrast, 5-HT$^+$ axons from ES cell grafts extended equally into white and gray matter regions of the host striatum. Thus, the difference between growth patterns of TH$^+$ and 5-HT$^+$ axons reflects characteristics that are typical of these mature CNS cell types.

We also observed large numbers of GFAP+ cells within ES cell grafts. The distribution of GFAP+ cells within graft regions corresponded to neuron-rich zones. GFAP-immunoreactivity is not donor cell specific and GFAP+ astrocytes were also present in the surrounding host tissue, so we cannot conclude that these cells are graft derived, as opposed to host-derived astrocytes that migrated into the grafts. Species-specific glial markers will be required to verify the origin of these GFAP+ cells.

Use of Blastula-Derived Stem Cells in Transplant Studies

By comparing grafts produced from two genetically distinct ES cell lines, we have further demonstrated that *in vivo* monoaminergic cell production is not due to unique characteristics of these particular cell lines. The E14TG2a cell line used in this study was developed to produce a transgenic mouse model of Lesch-Nyhan disease (10, 24). These cells contain a mutation of the gene encoding the purine salvage protein hypoxanthine-guanine phosphoribosyltransferase (HPRT). It is notable that these HPRT-deficient mice exhibit low levels of brain dopamine (10), but do not exhibit significant reductions in TH$^+$ neurons (24). By comparing HPRT-deficient ES cells with the HPRT-normal D3 ES cells in identical transplantation conditions, we did not find differences in the capacity of the cell lines to generate TH$^+$ and 5-HT$^+$ neurons. This confirms that the HPRT gene did not significantly bias the monoaminergic neuronal differentiation of cells in these grafts. Further studies are required to determine whether TH$^+$ neurons derived from HPRT-deficient ES cells have altered DA production.

In parallel to results reported here for ES cells, some EC cell lines can differentiate into terminal, nonproliferating neural phenotypes after pretreatment with RA and subsequent intracerebral transplantation (27, 33, 34, 56), and in some cases produce TH$^+$ cells (34, 56). In contrast to our present results, NT2 EC cells transplanted into immunodeficient mouse kidney capsule or muscle, or to subarachnoid space and superficial neocortex, produced tumors with heterogeneous morphology and rare neuronal differentiation (33). However, after transplantation into mouse caudate putamen, these EC cells gradually lost their proliferative capacity and produced immature neuron-like cells which stained for phosphorylated neurofilament and MAP2, with small nestin$^+$ regions within the grafts. Kleppner *et al.* (27) observed differentiated neuron-like cells which exhibited different patterns of innervation into the host brain depending on the region of the mouse brain in which they were implanted. In contrast to these results with EC cells, the ES cells in our present study do not seem to be dependent upon the site of transplantation for differentiation into neurons. Neurotransmitter-specific cell types from transplanted EC cells have been observed in some studies. For example, transplanted RA-treated P19 cells differentiated and expressed gamma butyric acid, neuropeptide Y, enkephalin, and somatostatin within the 6-OHDA- or ibotenic acid-lesioned adult rat striatum, although very few TH$^+$ cells were detected (34). Similar to our findings, untreated P19 cells transplanted to ibotenic acid-lesioned hosts grew to occupy the entire striatum, yet contained differentiated neurons and glia in similar proportions to RA-treated grafts. Wojcik *et al.* (56) observed a high density of TH$^+$ cells when the RA-treated EC cell line PCC7-S-aza-R-1009 was transplanted into the kainic acid-lesioned ventrobasal complex of the rat thalamus.

IM cell lines also exhibit a capacity to differentiate into a number of region-specific neuronal morphologies when transplanted into brain. The transplantation of these cells into neonatal brain resulted in differentiation into neurons and glia with region-specific morphology (5, 41, 46; 50). For example, RN33B cells (49), when transplanted into adult or neonatal rat hippocampus and cortex, exhibited morphologies similar to cells of the transplant site, suggesting that these IM cells respond to the local environment. This could have resulted from region-specific induction of precursor cells, and/or survival of differentiated cell types. Snyder *et al.* (50) also observed site-specific neural differentiation of v-myc-generated IM cell lines when transplanted into the neonatal mouse brain, as well as migration away from the implantation site. However, when transplanted into adult brain, Lundberg *et al.* observed that the plasticity of IM cells (generated from embryonic striatum or hippocampus) was more restricted: a majority of IM cells differentiated into glia in the adult environment (29).

GFE cells have also been transplanted into the adult brain, forming small grafts which exhibit some migration of cells away from the implantation site. After EGF-responsive progenitors isolated from the embryonic mesencephalon or striatum were transplanted into adult striatum, Svendsen *et al.* observed low cell survival within small grafts, with few differentiated cells expressing neuronal markers (52). In another study, FGF-2-responsive progenitors isolated from and transplanted back to the adult hippocampus displayed morphological characteristics typical of granule cells after

migrating into the dentate gyrus, though most cells remained localized near the injection track (13).

Currently, there is insufficient evidence to determine whether such cells develop through a sequence of states that correspond to those of normal embryogenesis. It is also difficult to determine the initial developmental status of GFE, IM, and EC progenitor cells. In particular, it is difficult to assess the exact genomic organization of IM cells, or how the loss of cell cycle control or chromosomal abnormalities affect the developmental processes of EC cells (17). Initial state, totipotency, and normal developmental and functional capacities are all known for the ES cells used in the present study. When inserted into embryos these cells are capable of normally differentiating into all cell types (3, 9, 36) and when cultured in the absence of LIF they can differentiate into a variety of cell types *in vitro,* including homeopoetic (25, 48), muscle (8, 45), and neural (2, 7) cell types. The experimental production of mature neuronal phenotypes from chemically or genetically modified cell lines as well as from normal ES cell lines suggests that neuronal developmental pathways may be accessible from many different antecedent states.

In contrast to results from many of the studies using EC, IM, and GFE cell lines cited above, transplanted ES cells in our paradigm did not differentiate into phenotypes corresponding to those of the implantation site. Due to their more undifferentiated initial state, it is possible that ES cells may not be responsive to as wide a range of signals as are slightly more differentiated cells. It is also conceivable that LIF expansion could influence differentiation. Although LIF does not restrict fates of ES cells when reimplanted into embryos (3, 9, 36), it is possible that a subtle predisposition toward certain fates would be masked by differentiation signals in early stage embryo but not after transplantation into the adult body. A more important factor may be differences of implantation procedure. In this study, implantation of ES cells as a single large bolus in a mature brain creates conditions in which donor cells are localized, cell densities are high, and the vast majority of cell–cell interactions are between ES cells, not between ES cells and host cells. This may have contributed to the apparent consistency of ES cell graft differentiation irrespective of graft placement (e.g., kidney capsule versus CNS). The majority of studies that have shown site-specific differentiation of other progenitor cells have involved implantation of cells into immature brains, implantation of cells that migrate out of the graft region, or implantation of smaller numbers of cells into more distributed sites. Transplantation of ES cells using these paradigms that increase donor cell/host cell interactions could resolve these questions.

Neural induction regardless of transplant site in our paradigm is consistent with recent evidence, suggesting that neuralization is a default pathway, and occurs spontaneously if pregastrula cells do *not* receive other inducing signals to form epidermal, mesodermal, or endodermal cells (20). This was first suggested by experiments showing that cells of the early gastrula ectodermal animal cap, that normally develop into epidermal tissue, all form neural tissue if dissociated (16). Bone morphogenic protein (BMP4) and activin have been implicated as the major inducers of epidermal differentiation during gastrulation. Ectopic application of BMP4 is sufficient to induce epiderm formation in dissociated animal pole cap cells (55), and homozygous knock-out mice lacking functional BMP receptor (BMPR1) die in gastrulation (21), a time when epidermis would otherwise form. Also, antagonists of BMP4 or activin signalling, such as noggin, follistatin, and chordin, which are produced in the Spemann organizer region, can induce the ectopic formation of neural tissue (19, 47, 57). The inactivation of the dominant activin receptor also induces neural differentiation (18). Thus, any manipulation that disrupts these epidermis-inducing signals results in neural differentiation. In our experiments, transplanting cells that have been dissociated and expanded at the pregastrula stage may disrupt the localized cell–cell communications which otherwise inhibit neuralization.

In conclusion, the ability to produce identified neuronal phenotypes from an initially totipotent state provides an experimental paradigm for further investigating the mechanisms underlying neural differentiation. The demonstration that neuronal differentiation occurs in ES cell grafts from a transgenic animal model of a neural disorder with a known genetic malfunction (HPRT-deficiency) also provides a new paradigm for study of the role of specific molecular defects on neural cell development. Finally, pathologies affecting the two classes of monoaminergic neurons produced in abundance in ES cell grafts have been implicated in congenital neurological disease, neuropsychiatric syndromes, and neurodegenerative conditions. The study of monoaminergic neurons derived from totipotent cells may provide information relevant to their associated developmental mechanisms.

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